



Generation of a monkey-tropic human immunodeficiency virus type 1 carrying *env* from a CCR5-tropic subtype C clinical isolate

Hiroyuki Otsuki, Mai Yoneda, Tatsuhiko Igarashi, Tomoyuki Miura*

Laboratory of Primate Model, Experimental Research Center for Infectious Diseases, Institute for Virus Research, Kyoto University, 53 Shogoin Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan

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ABSTRACT

Several derivatives of human immunodeficiency virus type 1 (HIV-1) that evade macaque restriction factors and establish infection in pig-tailed macaques (PtMs) have been described. These monkey-tropic HIV-1s utilize CXCR4 as a co-receptor that differs from CCR5 used by most currently circulating HIV-1 strains. We generated a new monkey-tropic HIV-1 carrying *env* from a CCR5-tropic subtype C HIV-1 clinical isolate. Using intracellular homologous recombination, we generated an uncloned chimeric virus consisting of at least seven types of recombination breakpoints in the region between *vpr* and *env*. The virus increased its replication capacity while maintaining CCR5 tropism after *in vitro* passage in PtM primary lymphocytes. PtM infection with the adapted virus exhibited high peak viremia levels in plasma while the virus was undetectable at 12–16 weeks. This virus serves as starting point for generating a pathogenic monkey-tropic HIV-1 with CCR5-tropic subtype C *env*, perhaps through serial passage in macaques.

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Introduction

Nonhuman primate models with human-like immune systems are often employed to evaluate the efficacy of candidate vaccines against acquired immune deficiency syndrome (AIDS). However, human immunodeficiency virus type 1 (HIV-1) infects humans or chimpanzees (*Pan troglodytes*) but not rhesus macaques (*Macaca mulatta*), the most widely used primate species in biomedical research (Gibbs et al., 2007). Experimental infection of macaques with simian immunodeficiency virus (SIV) or simian-human immunodeficiency virus (SHIV) has been used extensively to investigate HIV-1 infection *in vivo*. Pathogenic infection with SIV allows insight into the mechanisms of pathogenesis and provides information for development of novel vaccination strategies. However, due to the marked antigenic difference in viral proteins between HIV-1 and SIV, macaque models with SIV are not suitable for evaluating the immune response directed against HIV-1 (Javaherian et al., 1992; Kanki et al., 1985; Murphey-Corb et al., 1986). SHIV, a chimeric virus carrying *tat*, *rev*, *vpr* and *env* from

HIV-1 with an SIV genetic backbone, has been constructed and used widely to assess the immune response and pathogenicity directed against HIV-1 Env (Shibata and Adachi, 1992; Reimann et al., 1996; Harouse et al., 1999).

Highly pathogenic SHIV irreversibly depletes circulating CD4⁺ T-lymphocytes, and cause rapidly AIDS-like symptoms in infected macaques. These properties are, however, different from the vast majority of circulating HIV-1 or SIV isolates, and the discrepancy would be attributed to the viral co-receptor preference (Nishimura et al., 2004). Entry of HIV-1 into cells is mediated through the interaction of viral envelope protein with cellular CD4 and subsequent binding to either the CCR5 or CXCR4 chemokine receptor or both receptors. The vast majority of HIV-1 clinical isolates preferentially utilize CCR5 as the co-receptor for entry (Choe et al., 1996). The CXCR4-tropic or dual-tropic viruses that utilize both CCR5 and CXCR4 emerge during late stages in the disease course (Doranz et al., 1996; Feng et al., 1996).

In addition to the co-receptor usage, it is necessary to consider the variation of *env* gene in SHIV construction. Most HIV-1 strains currently circulating belong to group M, consisting of subtypes A–D, F–H, J, K and their recombinants, and are largely responsible for the global AIDS pandemic (Hemelaar, 2012). Most of early SHIVs are generated by utilizing genes derived from subtype B

* Corresponding author. Tel.: +81 75 751 3984; fax: +81 75 761 9335.
E-mail address: tmiura@virus.kyoto-u.ac.jp (T. Miura).

viruses, which comprise an estimated 11% of the global prevalence of HIV-1. By contrast, subtype C is the dominant subtype, accounting for almost 50% of global infections. Subtype C viruses do not share the antigenicity of Env as the main target of neutralizing antibodies with subtype B viruses (Choisy et al., 2004; Gaschen et al., 2002). The V3 loop region of the subtype C envelope is less variable than that of other subtypes (Kuiken et al., 1999), and mutations appear to accumulate in the C3 and V4 regions, which are targets of autologous neutralizing antibody responses in individuals infected with subtype C viruses (Moore et al., 2008, 2009). The structure of these epitopes is dissimilar between subtypes B and C (Gnanakaran et al., 2007). There are pathogenic SHIVs that encode CCR5 tropic subtype C env gene (Ndung'u et al., 2001; Ren et al., 2013; Song et al., 2006).

Conventional SHIV that encodes SIV sequence in 5' half of the genome has limited utility in the evaluation of cell-mediated immunity induced by a vaccine because it does not contain HIV-1 Gag in its genome; consequently, SHIV has different major epitopes for cytotoxic T lymphocytes (CTLs) known to be associated with lowering the plasma viral load in HIV-1 infection (Goulder and Watkins, 2004; Kiepiela et al., 2007). Recently, two major restriction factors were reported to block HIV-1 replication in monkey cells in a species-specific manner (Neil and Bieniasz, 2009). The restriction factor apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) protein is incorporated into viral particles and induces hypermutation in proviral DNA in target cells mediated by its cytidine deaminase activity (Sheehy et al., 2002). Macaque APOBEC3G proteins are counteracted by the SIV Vif protein but not by HIV-1 Vif (Mariani et al., 2003). The other major restriction factor that inhibits the viral replication cycle is tripartite motif 5 α (TRIM5 α) protein, which directly recognizes incoming viral capsid (CA) (Stremlau et al., 2004). HIV-1 CA can bind cyclophilin A (CypA), a ubiquitous cytosolic protein, to evade restriction by human TRIM5 α , whereas the CypA-binding activity appears to enhance TRIM5 α recognition in macaque cells (Berthouex et al., 2005; Keckesova et al., 2006; Stremlau et al., 2006). It is known that the host species barrier of Pig-tailed macaques (PtMs) (*Macaca nemestrina*) against HIV-1 is weaker than other macaques because they do not have the TRIM restriction (Brennan et al., 2008).

Based on these findings, derivatives of HIV-1 that has a remarkably different structure from the conventional SHIV were constructed by the introduction of minor genetic modifications into its genome to overcome the restriction factors in macaque cells. Hatziioannou et al. (2006) generated simian-tropic HIV-1 (stHIV-1) by replacing the entire *vif* gene of HIV-1 with that of SIVmac or HIV type 2. Kamada et al. (2006) reported the monkey-tropic HIV-1 (HIV-1mt) NL-DT5R, in which the CypA-binding motif of the CA protein is substituted by the corresponding sequence of SIVmac, and the entire *vif* gene is also substituted. Thippeshappa et al. (2011) generated HSIV-vif, a clone of HIV-1 by substituting the *vif* gene with that of a pathogenic SIVmac clone. These derivatives of HIV-1 established persistent infection in PtMs for months but were controlled thereafter (Hatziioannou et al., 2009; Igarashi et al., 2007; Thippeshappa et al., 2011). These monkey-tropic HIV-1 derivatives currently available are not CCR5-tropic; NL-DT5R and HSIV-Vif encode *env* from a CXCR4-tropic, and stHIV-1 encodes *env* from dual-tropic subtype B viruses.

In this study, we generated a new HIV-1mt strain carrying *env* from a CCR5-tropic subtype C HIV-1 clinical isolate. We employed intracellular homologous recombination (IHR) to produce the recombinant virus. Since the viral swarm generated by IHR did not show efficient replication in PtM primary cells, we conducted *in vitro* serial passages of the virus. Thus, we successfully generated a viral swarm that exhibited an enhanced replication capacity in PtM cells and established infection in PtMs with high peak

viremia comparable to the currently available monkey-tropic HIV-1 derivatives.

Results

Generation of a new HIV-1mt carrying CCR5-tropic subtype C *env* through IHR

We employed IHR to generate recombinant viruses (Fujita et al., 2013). First, we prepared DNA fragments by polymerase chain reaction (PCR) amplification of a region spanning the 5' long terminal repeat (LTR) to upstream of the V1/V2 region in *env* (nucleotide positions 1–6784 based on HXB2 numbering; accession number: K03455) using the plasmid DNA template encoding the full-length NL-DT5R proviral genome (fragment I in Fig. 1A). This fragment encodes a CypA-binding motif derived from the corresponding sequence of SIVmac239 to evade restriction from macaque TRIM5 α , and the entire SIVmac239 *vif* gene to counteract the macaque APOBEC3G. Second, a region spanning the *vpr* gene to the R region of the 3' LTR (nucleotide positions 5558–9625 based on HXB2 numbering) was amplified from the HIV-1 97ZA012 strain (fragment II in Fig. 1B). To increase the possibility to obtain a virus that can replicate in monkeys well, we thought that it was better to generate swarm viruses having variation without cloning. Resultant recombinant virus might fail to replicate normally if recombination occurred between fragments I and II that resulted in the 5' LTR of subtype B and the 3' LTR of subtype C. The discordance of the 3' and 5' LTR may disrupt successful translocation of the minus strand strong stop DNA to the plus strand genomic RNA during reverse transcription (Goff, 2007). To match the sequence of the 3' LTR to that of the 5' LTR, we prepared a third DNA fragment encoding a region spanning the 5' LTR to the middle of *gag* (nucleotide positions 1–1433 based on HXB2 numbering) from the proviral DNA extracted from HIV-1 97ZA012-infected cells (fragment III in Fig. 1B). Fragments I and II

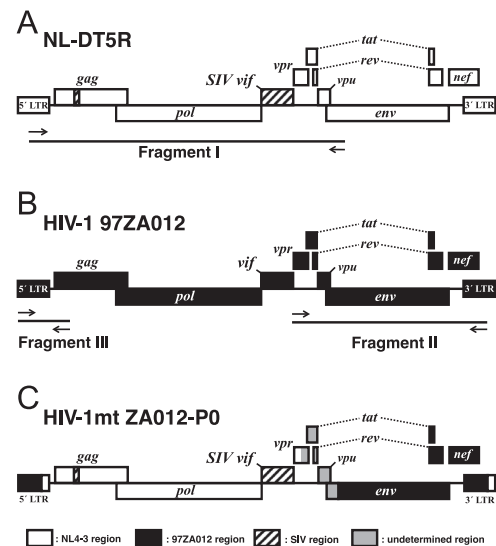


Fig. 1. Schematic representation of the genome organization of human immunodeficiency virus (HIV)-1 and monkey-tropic HIV-1 (HIV-1mt). Genome organizations of NL-DT5R (A), subtype C HIV-1 97ZA012 (B) and HIV-1mt ZA012-P0 (C) are depicted. The horizontal line represents DNA fragments I, II and III, used for intracellular homologous recombination. Fragment I encodes a region from the 5' LTR to *env* of NL-DT5R plasmid DNA. Fragment II encodes a region from the initiation of *vpr* to the R region of the 3' LTR of the HIV-1 97ZA012 strain. Fragment III encodes a region from the 5' LTR to upstream of the cyclophilin A-binding motif of the virus. Sequences from NL4-3 (open box), HIV-1 97ZA012 (filled box) and the SIVmac239 genome (diagonally striped box) are depicted. The gray box in HIV-1mt ZA012-P0 represents a gene that was not identified by direct sequence analysis.

had an overlapping region between the initiation of *vpr* to upstream of the *env* V1/V2 region, and fragments I and III had an overlapping region between the 5' LTR to upstream of the CypA-binding site.

These amplified DNA fragments (fragments I, II and III) were co-transfected into C8166-CCR5 cells that are permissive to CCR5-tropic HIV-1. On day 8 post-transfection, we observed the formation of virus-induced cytopathic effects (CPEs), indicating the generation of replication-competent recombinant virus. The new recombinant virus was isolated and designated HIV-1mt ZA012-P0.

To determine the genomic organization of HIV-1mt ZA012-P0, we subjected the viral RNA isolated from the culture supernatant to direct sequencing. We found that the virus carried sequences of the U5 region of the 5' LTR, *gag*, *pol* and *vif* derived from NL-DT5R and sequences of 3' half of *env*, *nef*, and *R* and the U3 region of the 3' LTR derived from 97ZA012 (Fig. 1C). First, the recombination breakpoint derived from fragments I and III was found to be located within the junction between the U5 and R region of the 5' LTR (nucleotide positions 551–605 based on HXB2 numbering). However, additional recombination breakpoints between fragments I and II, encoding the *vpr-env* region, were not identified due to multiple peaks at the same locations in the analyzed sequence chromatograms. This result suggested that HIV-1mt ZA012-P0 represented a swarm that might contain several variants with various recombination breakpoints.

Increased replication competence of HIV-1mt ZA012 through long-term *in vitro* passage in CD8⁺ cell-depleted pig-tailed macaque peripheral blood mononuclear cells (PBMCs)

We subsequently determined whether HIV-1mt ZA012-P0 replicates in CD8⁺ cell-depleted pig-tailed macaque peripheral blood mononuclear cells (PtM PBMCs), in which the parental

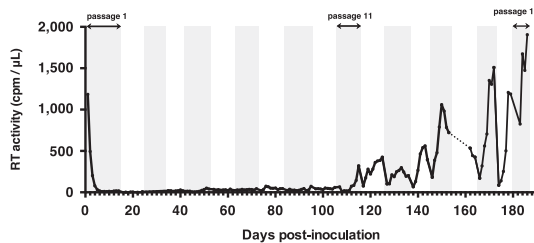


Fig. 2. Improved replication of HIV-1mt ZA012 throughout *in vitro* passages in CD8⁺ cell-depleted PtM peripheral blood mononuclear cells (PBMCs). HIV-1mt ZA012-P0 was used to spinoculate CD8⁺ cell-depleted PtM PBMCs, and virion-associated RT activity in the culture supernatant was monitored daily. Some of the infected cells were co-cultured with freshly prepared CD8⁺ cell-depleted PtM PBMCs. One period of passage was indicated in the shaded grey or white zones. The dotted line indicates data not available.

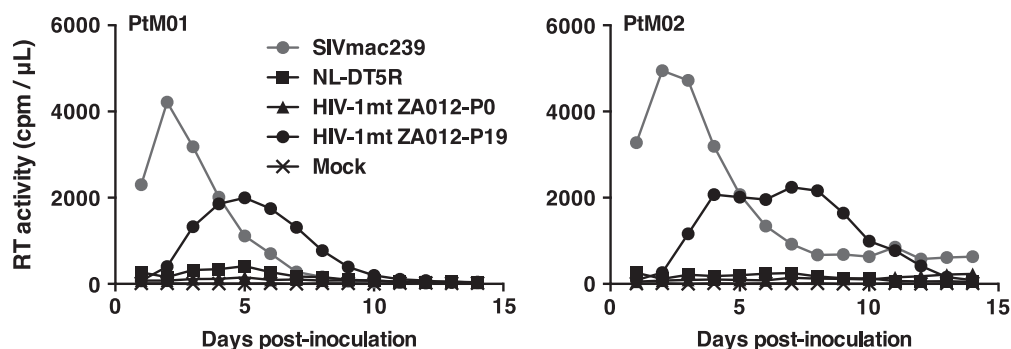


Fig. 3. Growth kinetics of HIV-1mt ZA012 in CD8⁺ cell-depleted PtM PBMCs. Growth kinetics of HIV-1mt ZA012-P0, HIV-1mt ZA012-P19, SIVmac239 and NL-DT5R were compared in PBMCs from two PtMs. Each virus was used to spinoculate CD8⁺ cell-depleted PtM PBMCs (MOI=0.1 TCID₅₀ per cell), and the virion-associated RT activity in the culture supernatant was monitored. The figure shown is representative of four independent experiments.

NL-DT5R replicated as described previously (Kamada et al., 2006). HIV-1mt ZA012-P0 from the culture supernatant of C8166-CCR5 was used to spinoculate CD8⁺ cell-depleted PtM PBMCs, and the virion-associated reverse transcriptase (RT) activity was monitored in the culture supernatant (Fig. 2); however, no RT activity was detected in the culture supernatant after passage 1 (Fig. 2).

Next we carried out *in vitro* serial passages to improve the replication competence of the virus as observed in the cases of HIV-1 (Freed and Martin, 1996; Willey et al., 1988). Infected cells were co-cultured with freshly prepared CD8⁺ cell-depleted PtM PBMCs every 1 or 2 weeks. Although detectable RT activity was not observed during 10 successive passages (passage 1–10), a low level of viral replication was confirmed by the CPEs of C8166-CCR5 cells co-cultured with PBMCs taken from the passage (data not shown). A detectable peak of viral replication (319 cpm/μL) was observed at 115 days after the first inoculation (passage 11), and replication was maintained following passages, eventually resulting in enhanced replication in PtM PBMCs (1900 cpm/μL in passage 19). The resultant virus, isolated from the culture supernatant of passage 19, was designated HIV-1mt ZA012-P19.

To evaluate the replication capacity of the virus, the replication kinetics of HIV-1mt ZA012-P19 were compared to those of the parental NL-DT5R and HIV-1mt ZA012-P0. Each viral stock was normalized by the number of infectious units per cell (in this case, a multiplicity of infection (MOI) of 0.1) and used to inoculate CD8⁺ cell-depleted PtM PBMCs isolated from two donor monkeys; virion-associated RT activity in the culture supernatant was monitored daily (Fig. 3). Although HIV-1mt ZA012-P19 exhibited a lower level of viral replication compared to that of SIVmac239, the virus showed more efficient replication than NL-DT5R and HIV-1mt ZA012-P0 in cells from both animals. Therefore, we successfully improved the replication capacity of the new HIV-1mt in PtM PBMCs by *in vitro* passaging.

Sequence analysis of HIV-1mt ZA012-P0 and ZA012-P19

It is likely that HIV-1mt ZA012-P0 acquired genetic changes and evolved to HIV-1mt ZA012-P19 through the serial passages in PtM PBMCs. To compare the genomic sequence of these viruses, we first performed single genome amplification (SGA) of viral RNA isolated from the culture supernatant to determine the nucleic acid sequences of the *vpr-env* region (nucleotide positions 5559–8795 based on HXB2) of HIV-1mt ZA012-P0. Subsequently, we identified the sequence of the region containing the expected recombination breakpoints generated by IHR between fragments I and II. Genetic analysis of 17 SGA clones revealed that these sequences had NL-DT5R sequences in the 5' end and HIV-1 97ZA012 sequences in the 3' end, with seven different recombination

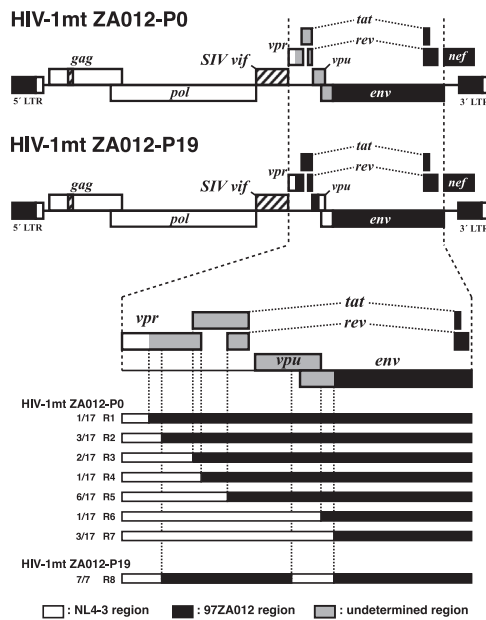


Fig. 4. Recombination breakpoints in HIV-1mt ZA012-P0 and ZA012-P19 genomes. The genome organizations of HIV-1mt ZA012-P0 and HIV-1mt ZA012-P19 are schematically represented (upper two diagrams). The region from the initiation of *vpr* to the end of *env* that included recombination breakpoint sites is depicted in the third diagram; the HIV-1mt ZA012-P0 (17 SGA sequences) or HIV-1mt ZA012-P19 (seven SGA sequences) are depicted (bottom). Sequences from HIV-1mt ZA012-P0 were classified into seven patterns of recombination breakpoints (R1 to R7). Sequences from HIV-1mt ZA012-P19 were classified into one recombination breakpoint pattern (R8). The numbers (left) indicate the numbers of sequences per analyzed sequence.

breakpoints in the region (Fig. 4). One recombination breakpoint was detected at nucleotide positions 178–187 of the *vpr* gene in 1/17 SGA sequences (5736–5745 in HXB2 numbering, recombination type R1) with 10 identical base pairs between NL-DT5R and 97ZA012. In addition to R1, we identified the following recombination types: the *vpr* gene in 3/17 SGA sequences (5760–5767; R2), the initiation of *tat* in 2/17 SGA sequences (5821–5839; R3), the end of the *vpr* gene in 1/17 SGA sequence (5852–5865; R4), the initiation of *rev* in 6/17 SGA sequences (5960–6000; R5), the end of the *vpu* gene in 1/17 SGA sequence (6357–6392; R6) and the upstream of V1/V2 of the *env* gene in 3/17 SGA sequences (6467–6491; R7). These results suggest that homologous recombination occurs in various sites with homologous sequences.

Next, seven SGA sequences were amplified from viral RNA isolated from the culture supernatant of PtM PBMCs infected with HIV-1mt ZA012-P19, and nucleotide sequences and recombination breakpoints were determined in the same manner. Unexpectedly, all the sequences of HIV-1mt ZA012-P19 had three recombination breakpoints in the region from the *vpr* to *env* genes (recombination type R8 in Fig. 4). The first breakpoint was located in the *vpr* gene (5760–5767), the second was located in the *vpu* gene (6194–6213), and the third was located in *env* (6467–6491) with the N-terminal portion of C1 region from NL4-3 sequence. Although the pattern of recombination breakpoint of the virus differed from those of HIV-1mt ZA012-P0, the first and third recombination breakpoints were identical to the recombination type of R2 and R7, respectively (Fig. 4). It is likely that HIV-1mt ZA012-P19 was generated from further recombination events that occurred in the middle of the *vpu* gene (6194–6213) between recombination type R2 and R7 of HIV-1mt ZA012-P0.

It is conceivable that the genome of HIV-1mt ZA012-P19 acquired several amino acid mutations associated with the enhanced replication in PtM PBMCs. Compared with the deduced

amino acid sequences in HIV-1mt ZA012-P0, HIV-1mt ZA012-P19 acquired substitutions from Lys to Arg at amino acid position 432 in Pol-RT and Asp to Glu at position 232 in Pol-IN that were in the NL-DT5R backbone. In addition, an amino acid substitution from Phe to Ser at 139 in Nef was found in HIV-1mt ZA012-P19 compared to 17 SGA sequences derived from HIV-1mt ZA012-P0. No nonsynonymous substitutions were identified in Gag and Vif, the proteins responsible for evading TRIM5 α and APOBEC3. Around the recombination break points in HIV-1mt ZA012-P19, the *vpr* and *vpu* genes keep each open reading frame and do not contain any mutations in the region derived from NL-DT5R, respectively. Furthermore, consensus amino acid sequence of HIV-1mt ZA012-P0 and -P19 were also identical in the regions derived from HIV-1 97ZA012, respectively. These facts suggest that recombination was occurred to keep these genes intact.

Phylogenetic analysis of *env* genes

It is likely that HIV-1mt ZA012-P0 generated by IHR in human C8166-CCR5 cells was a swarm carrying diverse *env* sequences of the parental HIV-1 97ZA012, which evolved to HIV-1mt ZA012-P19 through *in vitro* passages. To evaluate the *env* variants selected in C8166-CCR5 cells or primary PtM cells, we determined 22 sequences of HIV-1 97ZA012, 17 sequences of HIV-1mt ZA012-P0

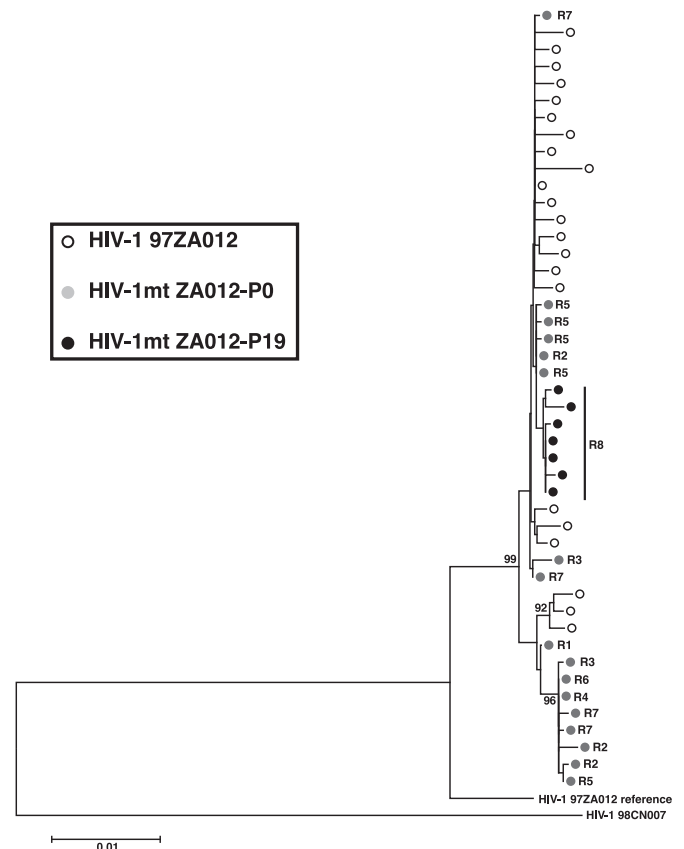


Fig. 5. Phylogenetic analysis of partial *env* sequences. A neighbor-joining phylogenetic tree was constructed from the partial nucleic acid sequences of *env* (nucleotide positions 211–2571 based on *env* of HXB2 numbering). The sequences of HIV-1 97ZA012 (white circle), HIV-1mt ZA012-P0 (grey circle) and HIV-1mt ZA012-P19 (black circle) were determined from SGA sequences. HIV-1 97ZA012 (accession number: AF286227) and 98CN007 (AF286230) reference sequences were obtained from the Los Alamos HIV sequence database (<http://hiv-web.lanl.gov/>). R1–R8 correspond to the patterns of recombination breakpoint types in Fig. 2. Bootstrap values were computed from 1000 bootstrap replicates, and only > 90% are shown at branches. The scale bar indicates the substitutions per site.

and seven sequences of HIV-1mt ZA012-P19 from SGA. Next, we conducted a phylogenetic analysis of the nucleotide sequences of the 3' terminal 2361 bp of each viral *env* derived from HIV-1 97ZA012 and shared by all variants of HIV-1mt ZA012-P0 and -P19 (Fig. 5). These sequences were divided into two clusters: the larger cluster included 19 sequences of HIV-1 97ZA012, 8 sequences of HIV-1mt ZA012-P0 and 7 sequences of HIV-1mt ZA012-P19; and the smaller cluster included 3 sequences of HIV-1 97ZA012 and 9 sequences of HIV-1mt ZA012-P0. Recombination types R2, R3, R5 and R7 (Fig. 4) were intermingled among the sequences of the two groups, suggesting that homologous recombination could occur in various *env* templates.

To compare the genetic diversity of *env* in these viruses, we computed the mean of all pair-wise distances between any two viral *env* sequences in each of the viruses. The computed diversity of *env* in HIV-1mt ZA012-P0 was 0.0038 ± 0.0025 (\pm standard deviation, SD), which was significantly lower than that in the parental HIV-1 97ZA012 (0.0044 ± 0.0021 ; $p < 0.05$). The computed diversity of HIV-1mt ZA012-P19 *env* was 0.0012 ± 0.00078 , which showed significantly lower variation compared to HIV-1mt ZA012-P0 ($p < 0.0001$).

Co-receptor usage of HIV-1mt ZA012-P19

To characterize co-receptor usage of HIV-1mt ZA012-P19 after long-term *in vitro* passage, we conducted an entry assay using TZM-bl cells with small molecule antagonists (Fig. 6). Viral infectivity of the CXCR4-tropic virus (NL4-3) was reduced in the presence of an increasing amount of the CXCR4 inhibitor, AMD3100, but was not affected by the CCR5 inhibitor, AD101. In contrast, the CCR5-tropic virus, SIVmac239, was inhibited in the presence of an increasing amount of AD101 but not by AMD3100. Similar to the results using SIVmac239, HIV-1mt ZA012-P19 exhibited sensitivity to inhibition by AD101 but resistance to AMD3100, indicating that the virus maintained its CCR5-tropism after the serial passage.

Replication of HIV-1mt ZA012 in pig-tailed macaques

Since HIV-1mt ZA012-P19 utilized CCR5 as a co-receptor and exhibited increased infectivity to primary cells of PtMs, we next assessed the *in vivo* replication capacity of the virus by experimental infection of PtMs. Two PtMs were inoculated intravenously

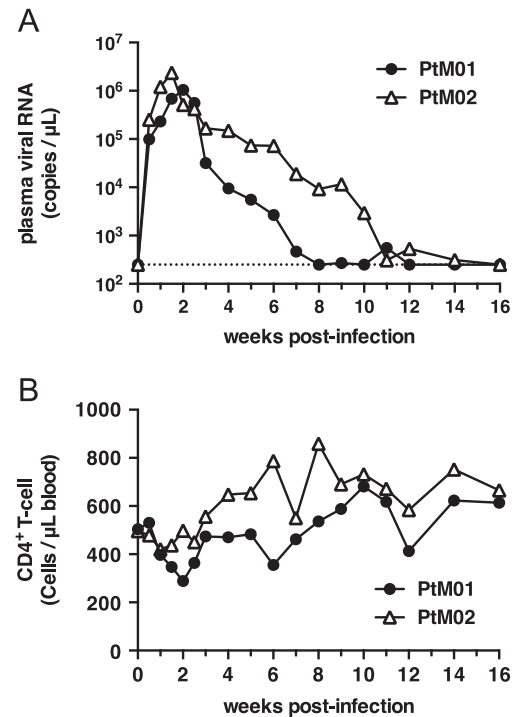


Fig. 7. HIV-1mt ZA012 infection of pig-tailed macaques. Two pig-tailed macaques were inoculated intravenously with HIV-1mt ZA012 (100,000 TCID₅₀), and the plasma viral RNA burdens (A) and circulating CD4⁺ T-lymphocytes (B) were monitored.

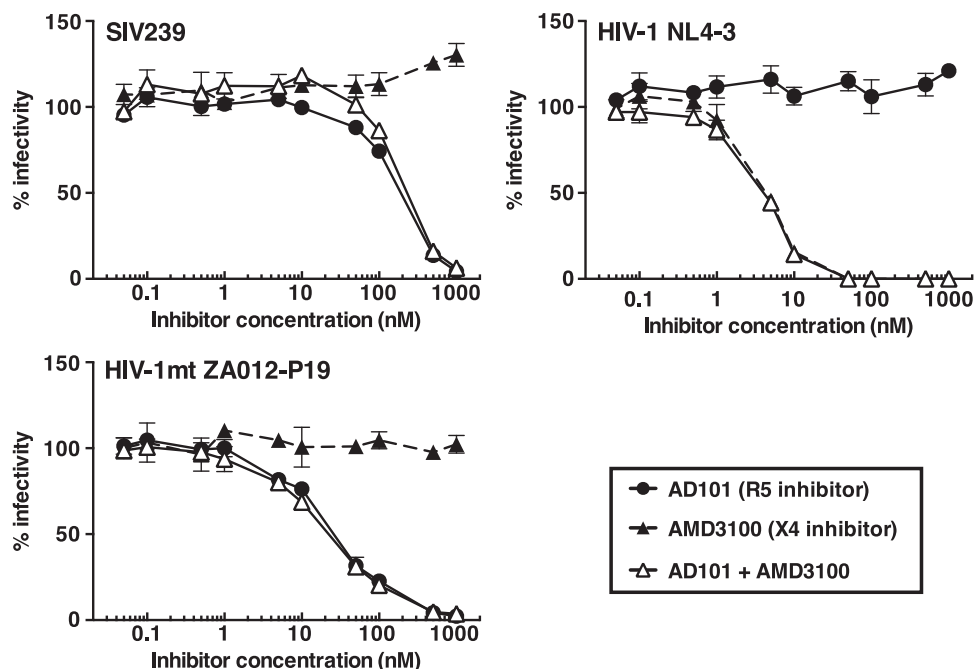


Fig. 6. Co-receptor usage of HIV-1mt ZA012-P19. Infectivity of HIV-1 NL4-3, SIVmac239 and HIV-1mt ZA012-P19 to TZM-bl cells was assessed in the presence of increasing amounts of AMD3100 (CXCR4 inhibitor), AD101 (CCR5 inhibitor) or both. The experiment was conducted in triplicate.

with 1.0×10^5 TCID₅₀ of the HIV-1mt prepared in PtM PBMCs, and plasma viral RNA burdens and the numbers of circulating CD4⁺ T-lymphocytes were monitored periodically (Fig. 7A). Plasma viral RNA loads in PtM01 peaked (1.0×10^6 copies/mL) at 2 weeks post-infection (wpi) and declined thereafter to levels below the detection limit at 8 wpi. PtM02 exhibited a peak plasma viral RNA burden (2.3×10^6 copies/mL) at 1.5 wpi and maintained more than 1×10^4 copies/mL by 9 wpi, but the viral load declined to levels below the detection limit at 16 wpi. The numbers of CD4⁺ T-lymphocytes in the circulation in both animals were not affected (Fig. 7B). Furthermore, we analyzed naive and memory populations of CD4⁺ T cells and no preferential depletion of circulating memory CD4⁺ T-lymphocyte was observed (data not shown).

Discussion

In this study, we used IHR to generate a new HIV-1mt carrying *env* from the CCR5-tropic subtype C HIV-1 clinical isolate. This recombination method has been used to generate infectious HIV-1 or SHIV by joining two linear DNAs in regions with completely identical sequences (Chen et al., 2000; Kalyanaraman et al., 1988; Kellam and Larder, 1994; Luciw et al., 1995; Srinivasan et al., 1989; Velpandi et al., 1991). Recently, we applied IHR to generate a replication-competent SHIV carrying subtype C *env* that was inserted within the *env* sequence of subtype B (Fujita et al., 2013). Here, we utilized the same method to generate HIV-1mt by replacing a coding sequence region from subtype B with that of a primary isolate of subtype C and investigated recombination breakpoints in detail by analyzing the sequences of the resultant viruses. We found seven variants with different recombination breakpoints that were located within overlapped sequences between fragments I and II. These variants were selected as replication-competent virus in C8166-CCR5 cells that maintained their variability, suggesting that IHR events occur frequently in cells co-transfected with DNA fragments. In addition, it appears that the length of identical sequence of as short as 8 bp is sufficient for IHR (recombination type R2 in Fig. 4). Furthermore, IHR is suggested to occur between various DNA templates, based on the phylogenetic analysis results that indicated intermingled types of recombination breakpoints among different *env* sequences.

To develop a virus that efficiently infects monkey cells, it is important to choose an *env* that mediates efficient entry to macaque cells. The Env proteins in most A–D subtypes of HIV-1 clinical isolates from infected individuals during the acute phase of infection do not mediate efficient entry using macaque CD4 receptors (Humes et al., 2012). In a preliminary experiment in C8166-CCR5 cells, we generated five strains of replication-competent HIV-1mt carrying *env* from subtype C HIV-1 clinical isolates, including 97ZA012, but only three were infectious to PtM cells (data not shown). The generation of SHIV 97ZA012 that can establish infection in rhesus macaques as described previously (Fujita et al., 2013) also suggested that Env of HIV-1 97ZA012 can generate recombinant viruses that are infectious to macaque cells.

The serial passage of HIV-1mt ZA012-P0 through PtM PBMCs resulted in the loss of variants with recombination breakpoints and led to the emergence of HIV-1mt ZA012-P19 variants with shared uniform mosaic breakpoints not detected before the passage (Fig. 4). It is possible that recombination type R8 was generated through additional recombination events within homologous sequences in the *vpu* region between variants with recombination type R2 and R7 because recombinant breakpoints located on *vpr* and *env* regions of the virus were identical to that of R2 and R7, respectively. This possibility of recombination between R2 and R7 is also supported by the previous finding that the AAAAA tract within the putative site of recombination is a recombination

hotspot during reverse transcription because the sequence facilitates template switching by pausing and dissociation of reverse transcriptase and results in frequent recombination (Quinones-Mateu et al., 2002).

HIV-1mt ZA012-P19 acquired three amino acid substitutions (K432R of Pol-RT, D232E of Pol-IN and F138S of Nef) through serial passages in PtM PBMCs, but the biological significance of these mutations remains undetermined. It has been reported previously that two amino acid substitutions (N222K and V234I) in the C-terminus of Pol-IN of NL4-3 could augment replication of HIV-1mt in cynomolgus macaque HSC-F and human MT4/CCR5 cells (Nomaguchi et al., 2013). A D232E mutation observed in this study was positioned near these two residues, which might be associated with increased replication in primate cells.

HIV-1mt ZA012 established infection in PtMs with the peak viremia reaching 1.0 – 2.3×10^6 copies/mL at 1.5 or 2 wpi (Fig. 7). In contrast, NL-DT5R exhibited low levels of replication in PtMs (at most 3.5×10^4 copies/mL at peak viremia) regardless of CD8⁺ cell-depletion, as described previously (Igarashi et al., 2007). Plasma viral RNA load at peak viremia in HSIV-vif infected newborn PtMs showed 0.5 – 1.0×10^5 copies/mL (Thippeshappa et al., 2011). The highest peak viral level has been achieved by stHIV-1 infection of PtMs, reaching 1.0×10^5 – 10^6 copies/mL at the peak (Hatzioannou et al., 2009). Although HIV-1mt ZA012 failed to persist its replication over 10 weeks, the replication capacity of the virus in the acute phase appeared to be comparable to or greater than known monkey-tropic HIV-1 isolates. The caveat is that HIV-1mt ZA012 was obtained through “autologous” cell passage.

The derivative of NL-DT5R was designed to counteract or evade restrictions by macaque TRIM5 α and APOBEC3G but not by interferon (IFN)-stimulated genes (ISGs). One of the IFN α -inducible host factors, tetherin, inhibits release of viral particles from infected cells (Neil et al., 2008). HIV-1 Vpu is able to counteract human tetherin activity but fails to downregulate this activity in macaque (Jia et al., 2009). On the other hand, unlike HIV-1 HXB2 or NL4-3, some strains of HIV-1 appear to antagonize macaque tetherin by its N-terminal transmembrane (TM) domain of Vpu (Shingai et al., 2011). It has been reported that replication of monkey-tropic HIV-1 could be controlled in macaque lymphocytes treated with IFN- α (Bitzegeio et al., 2013; Thippeshappa et al., 2013). Further investigations are required to determine whether HIV-1mt ZA012-P19 that encodes the N-terminal TM domain of Vpu, Env and Nef from subtype C could efficiently replicate in the presence of PtM tetherin or ISGs.

We generated the first CCR5-tropic HIV-1mt in the currently available derivatives of HIV-1 that can establish infection in macaques. NL-DT5R, HSIV-vif and stHIV-1 are infectious to PtMs, but these viruses are CXCR4 or CXCR4/CCR5 dual tropic. Several monkey-tropic HIV-1 isolates carrying CCR5-tropic *env* have been reported, but the viral replication was less efficient than NL-DT5R (Yamashita et al., 2008). The CCR5-tropic viruses preferentially infect memory CD4⁺ T-lymphocytes and efficiently replicate in effector sites *in vivo* (i.e., lymphocytes in the lung or gastrointestinal tract) (Brenchley et al., 2004; Mehndru et al., 2004; Okoye et al., 2007; Picker et al., 2004). Although we characterized co-receptor usage of HIV-1mt ZA012-P19 *in vitro*, further investigation is needed to determine whether the virus behaves similarly to CCR5-tropic HIV-1 isolates in patients *in vivo*.

In this study, we generated a new monkey-tropic HIV-1. The viral swarm HIV-1mt ZA012-P19 carries *env* sequences from CCR5-tropic subtype C HIV-1, and it successfully established infection in PtMs with a high peak viremia comparable or greater than the monkey-tropic HIV-1 strains currently available. Although the monkey-tropic HIV-1 requires further adaptation to improve its *in vivo* replication capacity, the virus potentially serves as a nonhuman primate model for AIDS, which reproduces infection with currently circulating HIV-1.

Materials and methods

Cells

293 T cells (DuBridge et al., 1987) were maintained in Dulbecco's Modified Eagle Medium (D-MEM; Wako, Osaka, Japan) supplemented with 10% (vol/vol) fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 1 mM L-glutamine. TZM-bl cells (Platt et al., 1998) from the NIH AIDS research and reference reagent program were maintained in D-MEM supplemented with 10% FBS, 1 mM L-glutamine and 1 mM sodium pyruvate. The human T-cell line, C8166-CCR5 (Shimizu et al., 2006) was maintained in Rosewell Park Memorial Institute 1640 medium (RPMI-1640; Invitrogen, Carlsbad, CA) supplemented with 10% FBS. PtM PBMCs from uninfected monkeys were isolated using the ficoll density gradient separation method. For this procedure, a mixture of 95% lymphocyte separation medium (Wako) and 5% phosphate buffered saline (PBS) was used as a separation solution as described previously (Agy et al., 1992; Frumkin et al., 1993). Residual erythrocytes were lysed in ACK lysing buffer (0.15 M NH_4Cl , 10 mM KHCO_3 , 1.0 mM $\text{EDTA} \cdot \text{Na}_2$). Depletion of CD8^+ cells was conducted with the magnetic-activated cell sorting (MACS) system (Miltenyi Biotec, Gladbach, Germany). Briefly, isolated PtM PBMCs were stained with phycoerythrin (PE)-conjugated anti-CD8 antibodies (clone SK1, BD Biosciences, San Jose, CA) and then labeled with anti-PE MicroBeads (Miltenyi Biotec). CD8^+ cells were removed using a magnetic column according to the manufacturer's instructions. PBMCs were cultured in RPMI-1640 supplemented with 10% FBS, 2 mM sodium pyruvate, 2 mM L-glutamine, 50 nM 2-mercaptoethanol and 40 $\mu\text{g}/\text{mL}$ gentamicin. PBMCs were stimulated with 25 $\mu\text{g}/\text{mL}$ Concanavalin A (conA) for 20 h and then cultured in the presence of 160 U/mL human recombinant interleukin-2 (IL-2; Wako).

Viruses

A stock of NL-DT5R virus was prepared from C8166-CCR5 cells transfected with a plasmid encoding full-length proviral DNA of NL-DT5R (pNL-DT5R) using the DEAE-Dextran/osmotic shock procedure (Takai and Ohmori, 1990). SIVmac239 (Kestler et al., 1988) stock virus was prepared from the culture supernatant of 293 T cells transfected with a plasmid encoding full-length proviral DNA of SIVmac239 with Lipofectamine (Invitrogen). CCR5-tropic subtype C HIV-1 clinical isolates including 97ZA012 were obtained from the NIH AIDS research and reference reagent program.

Generation of recombinant virus through intracellular homologous recombination

To generate recombinant virus by IHR, overlapping viral genomic DNA fragments were prepared by PCR amplification. A region spanning the 5' LTR to *env* was amplified from pNL-DT5R (GenBank accession number: AB266485) using the HIV-1-U3-NotI-F forward primer (5'-ATGCGGCCGCTGGAAGGGCTAATTTGGTCCCAAAG-3'; nucleotide positions 1–25 in NL-DT5R, and additional NotI site sequences) and the *env*-2R reverse primer (5'-CACAGAGTGGGGTAAATTTACAC-3'; nucleotide positions 6761–6784 in NL-DT5R). PCR was conducted with Expand long-range dNTPack (Roche Diagnostic, Basel, Switzerland). PCR conditions were as follows: 94 °C for 2 min followed by 10 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 8 min, 25 cycles of 94 °C for 15 s, 55 °C for 30 s, 68 °C for 8 min, with 20 s increments at 68 °C for each successive cycle and a final elongation period of 68 °C for 7 min (fragment I in Fig. 1A). Amplification of a DNA fragment spanning the initiation of *vpr* to the 3' LTR was derived from subtype C HIV-1 clinical isolates of the HIV-1 97ZA012 strain. Viral RNA was

isolated from culture supernatant using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized with Super Script III first-strand synthesis SuperMix (Invitrogen) using the OFM19-R reverse primer (5'-AGGCAAGCTTATTGAGGCTTA-3'; nucleotide positions 9604–9625 based on the HXB2 numbering). PCR amplification of the viral cDNA was conducted using HIV-1vpr-F forward primer (5'-AGATGGAA-CAAGCCCCAGAAGA-3'; nucleotide positions 5558–5579 in the HXB2 numbering) and OFM19-R reverse primer with the same conditions (fragment II in Fig. 1A). To prepare a fragment spanning the initiation of 5' LTR to the MA region of *gag*, proviral DNA was extracted from proviral DNA of subtype C HIV-1 isolate-infected C8166-CCR5 cells using DNeasy Blood & Tissue kits (Qiagen). The following amplification was conducted using HIV-1cladeC-U3-NotI-F forward primer (5'-ATGCGGCCGCTGGAAGGGTAAATTTACTCAAGAG-3'; nucleotide positions 1–24 in the HXB2 numbering plus NotI site sequences) and the PreSCA-R reverse primer (5'-AATCTATCCCATCTGCAGC-3'; nucleotide positions 1433–1414 in the HXB2 numbering) (fragment III in Fig. 1A). The PCR products were purified using QIAquick PCR purification kits (Qiagen).

Recombinant viruses were generated by means of IHR in the cell. PCR-amplified linear viral DNA fragments were co-transfected into C8166-CCR5 cells by the DEAE-dextran/osmotic shock procedure (Takai and Ohmori, 1990). After transfection, cells were maintained and passaged every 3 days. The culture supernatant was harvested upon observation of virus-induced CPE.

Virus titration

The infectious titer of the viruses was defined as the median tissue culture infectious dose (TCID_{50}) in TZM-bl cells as described previously (Li et al., 2005). Four-fold, serially diluted viral stock was used to inoculate TZM-bl cells (5000 cells per 200 μL of growth medium containing DEAE-Dextran at a final concentration of 12.5 $\mu\text{g}/\text{mL}$) in quadruplicate in flat-bottom 96-well plates. After incubation for 48 h at 37 °C, the culture supernatant was removed and the cells were treated with 50 μL of Cell lysis solution (Toyo-Inki, Tokyo, Japan) for 15 min at room temperature with shaking. Then, 30 μL of the cell lysate were transferred to F96 MicroWell plates (Thermo Fisher Scientific, Roskilde, Denmark), and the relative luminescence units (RLU) after adding 50 μL of luciferase substrate (PicaGene, Toyo-Inki) to each well was determined using a microplate reader (Mithrus LB940, Berthold Technologies, Bad Wildbad, Germany). Viral infectivity was measured in RLU, and positive wells were defined as $\text{RLU} > 2 \times \text{background}$. The TCID_{50} was calculated as described previously (Reed and Muench, 1938).

Viral growth kinetics in pig-tailed macaque PBMCs

PtM PBMCs were isolated from two uninfected animals and CD8^+ cells were depleted as described above. Two days after stimulation with Concanavalin A (25 $\mu\text{g}/\text{mL}$), 2.5×10^5 cells of CD8^+ cell-depleted PtM PBMCs were inoculated with 2.5×10^4 TCID_{50} of viral stocks by spinoculation (O'Doherty et al., 2000) at 1200g for 1 h at room temperature. After washing with PBS, the infected cells in 200 μL of culture medium were cultured in round-bottom 96-well plates at 37 °C. The upper 150 μL of culture supernatant without aspirating cells in the bottom of the well was exchanged with fresh medium everyday. The harvested supernatant was stored at –20 °C prior to measure the activity of RT associated with virions.

RT assay

The virion-associated RT activity in culture supernatant was monitored as described previously (Willey et al., 1988). Briefly,

6 μ L of culture supernatant were combined with 30 μ L of RT reaction cocktail [50 mM Tris-HCl, 75 mM KCl, 10 mM dithiothreitol, 4.95 mM MgCl₂, 10 mg/mL polyA RNA, 5 mg/mL oligo-dT₂₀, 0.05% NP40] and 1.66 \times 10⁴ Bq equivalent α ³²P-dTTP (PerkinElmer, Waltham, Massachusetts, USA) and incubated at 37 °C for 2 h with gentle agitation. Next, 3 μ L of incubated mixture were blotted onto DE81 ion exchange cellulose paper (GE healthcare, Buckinghamshire, UK). After four washes with 2 \times saline sodium citrate (SSC), the residual radioactivity from synthesized DNA was counted using a liquid scintillation counter.

Single genome amplification (SGA)

SGA of the region spanning the initiation region of *vpr* to the end of the *env* gene was conducted as described previously (Salazar-Gonzalez et al., 2008). Synthesized viral cDNA was end-point diluted and then subjected to nested-PCR. First-round PCR was conducted with KOD-FX (TOYOBO, Osaka, Japan) in a total of 20 μ L of reaction mixture, using the SGA-16F forward primer (5'-TGCAGCAGAGTAATCTTCCACTACAGG-3'; nucleotide positions 5260–5283 in NL-DT5R) and the SGA-OFM19R reverse primer (5'-AGGCAAGCTTTATTGAGGCTTAAGCAGTGG-3'; 9771–9800 in NL-DT5R). The first-round PCR conditions were as follows: 94 °C for 2 min, followed by 35 cycles of 98 °C for 10 s, 63 °C for 30 s and 68 °C for 5 min. Second-round PCR was performed using 1 μ L of the first-round PCR product using the SGA-17F forward primer (5'-AGAAGAGACAATAGGAGAGGCCTTCGAATG-3'; 5610–5639 in NL-DT5R) and the SGA-2.5R reverse primer (5'-AAAGCAGCTGCT-TATATGCAGCATCTGAGG-3'; 9673–9702 in NL-DT5R). The second-round PCR conditions were the same as those in the first-round PCR. Amplification of the target sequence was confirmed with agarose gel electrophoresis. According to a Poisson distribution, when a positive ratio of amplification from diluted cDNA is < 30% in multiple replicate PCR reactions, the amplicons are predicted to be amplified from one-copy of template with the probability of > 80%. The single genome amplicons were purified before sequence analysis.

Genomic analysis

Sequence analysis was performed using the BigDye terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the ABI PRISM 3130xl genetic analyzer (Applied Biosystems). The 3'-terminal 2304 nucleotide sequences of *env* were aligned using the Clustal X software (Thompson et al., 1997). A neighbor-joining phylogenetic tree (Saitou and Nei, 1987) using Kimura's two-parameter model (Kimura, 1980) was constructed using MEGA 5 software (Tamura et al., 2011), and bootstrap values were computed from 1000 bootstrap replicates (Felsenstein, 1985). Pair-wise distances between any two nucleic acid sequences of the 3' terminal 2361 bp of each viral *env* within the parental HIV-1 97ZA012, HIV-1mt ZA012-P0 and HIV-1mt ZA012-P19 were calculated with Kimura's two-parameter model (Kimura, 1980) by using MEGA 5 software (Tamura et al., 2011). The statistical significance between each viral pair-wise distance was calculated with Student's *t* test using GraphPad Prism software (San Diego, CA, USA).

Co-receptor usage assay

Employing a previously reported method (Nishimura et al., 2010) with minor modifications, co-receptor usage of viruses was determined using the small molecule antagonists, AD101 (Trkola et al., 2002) provided by Dr. Julie Strizki (Schering-Plough Research Institute, Kenilworth, NJ) and AMD3100 (Sigma-Aldrich, St. Louis, MO) (Donzella et al., 1998). Briefly, freshly trypsinized TZM-bl cells (5000 cells per 100 μ L of growth medium containing

DEAE-Dextran at a final concentration of 12.5 μ g/mL) were seeded in flat-bottom 96-well plates. The cells were incubated with 50 μ L of co-receptor antagonists at final concentrations ranging from 0.1 nM to 1000 nM for 1 h at 37 °C and inoculated with 100 TCID₅₀ of replication-competent virus in triplicate. After incubation for 48 h at 37 °C, luciferase activity was measured, and the percent infectivity relative to that measured in mock-treated wells was determined.

Experimental infection of pig-tailed macaques with HIV-1mt ZA012

HIV-1mt ZA012 challenge stock was prepared from culture supernatant of PtM PBMCs infected with HIV-1mt ZA012-P19. The virus was titrated with PtM PBMCs as described previously (Fujita et al., 2013). Two pig-tailed macaques, PtM01 and PtM02 aged 7 and 6 years, respectively, were intravenously inoculated with 1.0 \times 10⁵ TCID₅₀ of HIV-1mt ZA012. Plasma viral RNA loads were measured with TaqMan real time RT-PCR as described previously (Miyake et al., 2006) with minor modifications; RT-PCR was conducted for HIV-1 *vpr* amplification using the NM3rNvpr-F forward primer (5'-CAGAGACCAAGGCCACAG-3') and NM3rNvpr-R reverse primer (5'-GTCTAACAGCTTCACTCTTAAGTTCCTCT-3'). PCR products were detected with a labeled probe, NM3rNvpr-T (5'-Fam-AGGGAGCCATACAATGAATGGACACT-Tamra-3'; Perkin Elmer). Animal experiments were conducted in the biosafety level 3 animal facility, in compliance with institutional regulations approved by the Committee for Experimental Use of Nonhuman Primates of the Institute for Virus Research, Kyoto University, Kyoto, Japan.

Flow cytometry

To enumerate CD4⁺ T-lymphocytes, and memory and naïve CD4⁺ T-lymphocytes, whole blood samples were stained with fluorescently labeled mouse monoclonal antibodies. Anti-CD3 (clone SP34-2) conjugated with Pacific Blue, anti-CD4 (clone L200) conjugated with PerCP-Cy5.5, anti-CD8 (clone SK1) conjugated with APC-Cy7, anti-CD20 (clone L27) conjugated with FITC and anti-CD95 (clone DX2) conjugated with APC were purchased from BD Biosciences, and anti-CD28 (clone CD28.2) conjugated with PE was purchased from eBioscience (San Diego, CA). CD28^{high}CD95^{low}CD4⁺ or CD28^{high/low}CD95^{high}CD4⁺ T-cell subsets were considered as naïve or memory CD4⁺ T-lymphocytes, respectively (Pitcher et al., 2002). The absolute number of lymphocytes in the blood was determined using an automated hematology analyzer, KX-21 (Sysmex, Kobe, Japan).

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